A clinical perspective on the 2016 WHO brain tumor classification and routine molecular diagnostics

van den Bent, Martin J ; Weller, Michael ; Wen, Patrick Y ; Kros, Johan M ; Aldape, Ken ; Chang, Susan

Abstract: The 2007 World Health Organization (WHO) classification of brain tumors did not use molecular abnormalities as diagnostic criteria. Studies have shown that genotyping allows a better prognostic classification of diffuse glioma with improved treatment selection. This has resulted in a major revision of the WHO classification, which is now for adult diffuse glioma centered around isocitrate dehydrogenase (IDH) and 1p/19q diagnostics. This revised classification is reviewed with a focus on adult brain tumors, and includes a recommendation of genes of which routine testing is clinically useful. Apart from assessment of IDH mutational status including sequencing of R132H-immunohistochemistry negative cases and testing for 1p/19q, several other markers can be considered for routine testing, including assessment of copy number alterations of chromosome 7 and 10 and of TERT promoter, BRAF, and H3F3A mutations. For "glioblastoma, IDH mutated" the term "astrocytoma grade IV" could be considered. It should be considered to treat IDH wild-type grades II and III diffuse glioma with polysomy of chromosome 7 and loss of 10q as glioblastoma. New developments must be more quickly translated into further revised diagnostic categories. Quality control and rapid integration of molecular findings into the final diagnosis and the communication of the final diagnosis to clinicians require systematic attention.

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Review. A clinical perspective on the 2016 WHO brain tumor classification and routine molecular diagnostics in glioma.

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Dear colleagues,

We were pleased with the comments we received on our manuscript

“A clinical perspective on the 2016 WHO brain tumor classification and routine molecular diagnostics.”

Please find attached the revised version of our manuscript, revised according the comments and suggestions of the reviewers.

We have answered all comments raised by the reviewers point by point and submitted this together with the modified version, and we have included both a track changes version and a final version with all changes implemented.

No part of this manuscript has been published before nor is it under the consideration of another journal. All authors have read and approved the manuscript. No figures or tables were taken from another journal.

The total word count is now 7750 words. Figures have now been uploaded as .tif files, one combination figure was made in order to reduce the total number of figures and tables to 6.

We look forward to receiving the comments on this revised version.

With kind regards, on behalf of my co-authors,

Martin J van den Bent

We appreciated the thorough reviews we received, which all address important issues. Hereunder we reply to their comments point by point. :

Reviewer #1: The authors present a review of the new WHO Classification of Tumors of the Nervous System from the perspective of the clinician-user. The review focuses on adult gliomas and the integration of molecular diagnosis and histopathological diagnosis in the final diagnosis of the several entities. The review is a critical review with the opinion of the authors on different issues; therefore, one may disagree with the authors' opinions. Nevertheless, the review provides insights and perspectives on several aspects of tumor classification and guidelines for the readership.

The WHO has a mission to propose guidelines universally that, due to the differences in technological advances and resources between countries, socialized versus insurance-based medical systems, academic and private medical practices, may lead to disparities in implementation of these guidelines. Despite these caveats, the 2016 WHO classification of brain tumors provides a modern integrated classification with several diagnostic tools that can be used by the great majority of pathologists either in their own practices or in tertiary referrals centers.

As the authors have emphasized, any tumor classification is a moving target since new data may appear at the moment of its compilation. The WHO series of classification of tumors are assembled on evidence-based data and common-sense of their users. This sometimes takes time. Therefore, it is not surprising that while some users are still trying to understand the new proposals and changes of the 2016 classification, others are already proposing new modifications like the authors.

As commented before, one may have different opinions than those presented by the authors, and this reviewer has several of them. But these are not a point for review; they are differences of opinions. However, one point should be addressed by the authors. The 5 examples illustrated by the authors with neuroimaging and the results of the molecular diagnosis are a proof that neuro-oncology has to be practiced in a multidisciplinary manner from the review of the clinical presentation, the interpretation of imaging, neurosurgical approach, and integrated histopathological and molecular diagnosis. The way the authors choose to illustrate the cases minimize the value of histopathological diagnosis as the initial step for the pathological diagnosis and a guide for molecular diagnostics. Also, it minimizes the need for multidisciplinary tumor board review for each individual patient in a systematic manner.

A: We thank the reviewer for the summary, and of course if anything, the used MR images emphasize the need for multidisciplinary boards: they shows the value of re-examining the MR images when more diagnostic information is received, be it pathological or molecular. We have now emphasized that more clearly in the manuscript. We could have added microscopy to the images, but since clinicians are more involved in the review of MR images we choose not to.

Reviewer #2: The abstract reads a little clumsy. E.g. " Recent data have shown that analysis of genetic lesions allow a better prognostic and predictive classification of diffuse glioma." NOt sure this is the main point to start with; it's about diagnostic accuracy, not prediction or prognosis.

A: This is indeed the heart of the matter, and a key element where the discussion on how to classify tumors is about. But, diagnostic accuracy is not all there is, a diagnosis can be accurate but not informative if it does not inform about outcome. The current classification is also one that is better suited for therapeutic recommendations, but we agree this is not about true prediction and more about treatment selection. The text has thus been modified accordingly, with some additional changes to meet the word limit.

There is some claim to focus on adults, but the authors then continue with the lesion from pediatric GBM and also include medulloblastoma. It should be consistent. The speculative part (chromosome 7 gain and 10 loss patients to be considered as molecular GBM), IDH mut GBM to be renamed and the desire for a rapid transition from new findings into a classification is not harboring the same level of evidence as the prior statements and exceeds WHO CNS 4+; therefore I would leave the abstract for the facts from the WHO interpretation and put this less solid part into the main text. From this reviewer's perspective there would be enough to tell about the clinical implications of the classification and no need for "political statements in the abstract". Along the same lines, the classification process should be stringent and this does not allow to jump on every new finding.

A: In view of the comments we received we have deleted the section on medulloblastoma, which was indeed (and deliberately) superficial. The 7+/10q loss data are currently considered strong enough to be taken into consideration for treatment decisions by the many clinicians that have access to this type of diagnostics. In fact, this notion is also present in the Blue Book texts on low grade astrocytoma (page 26), AOA (page 76)and AOD (page 74). This is an area where the knowledge has been accumulating, for which reason we write in the manuscript: "then the WHO classification could consider going beyond the IDHwt diagnoses". It is also left as a consideration in the abstract, but to meet the comment we have rephrased the first 'should' into 'could. Indeed, when it comes to individual patients we cannot wait when deciding on treatment. With respect to the political statements, this has really been an issue.

In the main text 'The Genotype trumps the histological phenotype':

The statement "The demonstration that a molecular correlate of oligoastrocytoma does not exist, consistent with large differences in outcome of anaplastic oligoastrocytoma" is not quite correct and not quite reflecting the cited references. The main message is molecular separation and dependent on the pathologist. It is not gone, because there are differences in interstudy comparisons. It might be useful to check another early reference on that topic (Wiestler et al. ANP 2014, ATRX loss refines the classification of anaplastic gliomas and identifies a subgroup of IDH mutant astrocytic tumors with better prognosis).

A: ATRX mostly helps identifying the IDHmt but 1p/19q intact anaplastic glioma. The quoted manuscript is on the NOA4 study on anaplastic glioma. Interestingly, the same authors made clear in another manuscript on NOA4 (Wiestler et al, Integrated DNA methylation and CNA, Acta Neuropathologica) 2014) that 24 of investigated 115 NOA4 tumors were CIMP negative and molecularly resembling glioblastoma. The paper quoted by the reviewer primarily identifies with ATRX staining the IDHmt 1p/19q intact tumors, and thus the better prognosis of these tumors compared to the glioblastoma like cases (and the slightly less favorable outcome compared to co-deleted cases). Since ATRX mutations are not present in all IDHmut 1p/19q non-codeleted tumors and has at present not been associated with outcome within the subgroup of these IDH mutated 1p/19q intact tumors, we do not think this reference add to this review. Of note, these manuscripts are also supporting the identification of 7+/10q LOH tumors because of their poor outcome. We were already quoting this reference, we now also quote it in the section on 7+/10q LOH tumors

Consider medulloblastoma. It is now presented as a major focus, but discussed only very superficial.

A: We only entered the remark on medulloblastoma to acknowledge the molecular change that occurred here, without the intent of going into details; obviously the focus is on adult glioma. It was not intended as a major focus, and also in view of the comments of the other reviewers we have deleted it.

Further below a confusing statement is "One solution would have been to add to the 2016 classification the categories of (anaplastic) oligodendroglioma, IDHwt, as a way out of difficult to diagnose cases that undoubtedly will surface. The use of NOS for childhood oligodendroglioma without 1p/19q loss and IDH mutations is confusing, as in adults NOS restricted to those cases where testing was not possible or was non-conclusive." Although this reviewer tries to understand, what the authors want to say, it may need some context for the general reader.

A: We have tried to clarify this sentence to make it better understandable.

The "political" statements on the turn of cycles for the WHO classification is not accurately reflecting the present situation and would be better placed at the end. I have difficulties to understand, why the authors criticize the cycles although these are given by the WHO process, plus efforts exist to find more rapid updates, in which at least some of the autors are involved. A separate classification for sure would not solve the issue.

A: This is indeed an issue, we do understand the existence of a WHO process, but we do not agree with the fact that that process in itself governs the timing of revisions, and not the emerging data. This is simply not a patient centered approach, and this is perceived by many as an area of friction.

The genes suggested for testing should be prioritized as some are integral part of the classification and some are interesting (and relevant like MGMT), but some like EGFR or BRAF and others are nothing but a trial target at this given moment, which alone should not place it into the context of a clinical WHO commentary.

A: indeed, many of these these genes are not present in the WHO classification but based on recurrent questions we felt the discussion of genes that are proposed for testing in daily clinics was relevant for this review.

One caveat is regarding the integration of imaging. It should be stated, in which cases and what fore this is necessary. Sampling error (which is less of an issue)...

A: We fully agree with this point. In general, this should be done in all cases: MRI images should always be consistent with the diagnosis, if not this should induce suspicion and more scrutiny. This is now more clearly stated in the manuscript.

Reviewer #3: This is a review of the new WHO classification for CNS tumors. This is a topic that warrants discussion among clinical neuro-oncologists as it has the potential to dramatically change the therapeutic approach to several entities (eg. IDHWT low grade gliomas). However, this review reads as a criticism of the new classification and makes recommendations about adding or subtracting molecular testing depending upon the circumstances. This is potentially very dangerous, given that this new scheme is only a few months old, few people are completely facile with all the nuances of this new approach and that additional testing may or may not be available to everyone - a major reason the current update was limited to examine IDH and 1p19q status. Thus, the focus/purpose of the review is murky and not well organized which dramatically limits its usefulness to the reader.

A: we understand the concern of the reviewer, but it is no secret this revision has been subject to a debate and this debate is not resolved; moreover new developments will raise new issues. I have full confidence in the critical minds of the readership of Neurooncology, and in our view this journal should not avoid such discussion.

1. On page 4 the authors state that the use of NOS is not recommended if IDH testing was inconclusive or unavailable. Really? They don't propose an alternative. There will always be times when testing is inconclusive - for a variety of reasons - so what is the option in view of the fact that knowledge of IDH status is co side red parse punt to treatment planning? At least NOS makes clear that the pathologist attempted (or thought o) the testing but results were not available. This seems reasonable to me, and was suggested after enormous consideration by the most experienced tumor neuropathologists in the world.

A: To our defense, we took this 'not recommended' for NOS from an presentation given by one of the leading participants to the WHO 2016 revision. When checking the text, we indeed found no similar remark, neither in the blue book nor in the 2016 review by Louis. We have therefore deleted this sentence.

2. The discussion on page 5 in Disappearing Glioma Entities presumes access to the WHO 2016 text. The diagnosis of gliomatosis cerebri was always based on the imaging which is something a clinician would

always consider when deciding upon treatment. This reader is not sure how suggesting that the term be retained by clinicians is helpful.

A: Indeed, gliomatosis has always been a radiological diagnosis, and clinicians continue to consider the spread of tumor when deciding on radiotherapy. It has therapeutic consequences, but it remains ill defined. We fully agree though that it is no longer part of the WHO classification.

3. On the one hand, the authors recommend completely different nomenclature for GMB, IDH mutant, suggesting they are called astrocytoma grade IV IDH mutant. They make the point about prognosis but there have been many know factors which affect GBM survival (such as age), but we didn't call the tumor by a different name in different age groups to reflect that difference. On the other hand, at the end of the paper they actually suggest that IDH testing be limited to patients aged 15-55! This is in direct contradiction with the new criteria and their argument on page 6-7 that IDH should be at the "heart" of the astrocytoma diagnosis. This seems enormously confusing and counterproductive to make these suggestions.

A: The reviewer touches upon critical points. With respect to the remark on age and other prognostic factors, the WHO classification considers tumor morphology, and now includes molecular factors. They do not and never did consider clinical factors, and rightly so. What we propose is based on molecular factors, and part of the classification. We therefore do not see the argument clinical factors, which are clearly outside the testing of the tumor sample.

With respect to the proposed age limit for IDH: we did not propose to limit testing to the age of 15-55 years, we wrote "Testing for IDH is part of routine diagnostics, but it seems reasonable to limit routine testing to an age range of 15 to 55 years, for example, and test beyond that only on clinical indications (e.g. in all adult grade II and III glioma, and in case of a hemispheric astrocytoma in a 13 year old).";
acknowledging that testing beyond is useful on indication. So basically the question is what the diagnostic yield will be to test for IDH mutations in case of a tumor in a 63 year old patient with the histological diagnosis of a glioblastoma and an MR lesion consistent with a glioblastoma. As with other diagnostic tests, some considerations about diagnostic yield makes sense as opposed to simply testing without further considerations. We have made the proposal for the upper age range for routine testing testing somewhat broader as there are no data to support a strict cut off.

4. The figure legends for the pictures of figures 3 and 4 are reversed and it's not clear in the text where they want which picture.

A: we apologize, while editing the text we failed to update the figure legends, this is now corrected (on top, two of the images have been combined).

5. The brief sections on medulloblastoma, ependymoma and the "WHO cycle" seemed to be dropped into the middle of this paper. The authors then cycle back to the gliomas. This is confusing and seems

disorganized. All the glioma information should be together. One could argue that the other tumors could even be eliminated from this discussion.

A: as mentioned above, we intended only to touch upon medulloblastoma, and we have this section now removed. We bought some more background information on the status of molecular testing of ependymoma, which is after all a subgroup of the glioma.

6. On page 9, one could argue that clinicians have long ago adopted some aspects of molecular classification, especially 1p19q loss. I think that this version of WHO is actually catching up to the clinical approach to these patients - not the other way around.

A: We fully agree, and would deplore the situation in which the clinically used classification would become distant from the official WHO classification.

7. Given the current state of glioma therapeutics, it is unclear what the additional testing for TERT and chromosome 7+/10q- adds, given that many clinicians already view lower grade gliomas that are IDH WT as having a poor prognosis and moving to treat these patients. At this time, the additional information of further poor prognostic features would t change the treatment decision. This doesn't mean that further testing isn't appropriate within clinical trials or research environments, but to call for routine use of these features seems a bit over the top (pages 12-13).

A: This in fact becomes more and more part of the clinical approach to these patients, we do not think this is really over the top. We have rephrased the sentence in the abstract with recommendations for testing, indeed suggesting that this should be done routinely would be a bridge too far.

8. The language needs a lot of work. There are numerous spelling and grammatical errors (fossa posterior). First line, page 9 has the wrong reference (23); I don't know if other references are wrong. Page 17, third line from bottom saying that the FISH is "positive" is confusing here when trying to describe the pitfalls of partial deletion. There is a lot of redundancy which is also confusing and the authors do contradict themselves in multiple places - some of which I've listed above, but most importantly they recommend additional testing in most places and not even the basic testing in many others.

A: Ref 23 is on the use of methylation arrays to distinguish between poor prognosis and good prognosis fossa posterior ependymoma, with similar histological appearance. This reference is checked and correct. Of note, this part of the text has been revised and ref nr have been changed. We have rephrased 'positive' by 'loss' to avoid misunderstanding. The text was prepared with several native speakers, if errors still exist the corresponding author is to be held accountable for that. We do not agree we recommend 'not even the basic testing in many others', as mentioned above we try to be cost efficient in the use of resources and refrain from testing in cases where any patient benefit is quite unlikely.

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Review. A clinical perspective on the 2016 WHO brain tumor classification and routine molecular diagnostics.

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Abstract

The 2007 WHO classification of brain tumors did not use molecular abnormalities as diagnostic criteria. Studies ~~Recent data~~ have shown that ~~analysis of~~ genoetyping ~~ic lesions~~ allow a better prognostic ~~and predictive~~ classification of diffuse glioma with improved treatment selection. This has resulted in a major revision of the WHO classification, ~~('WHO 2016')~~ which is now for ~~the~~ adult diffuse glioma centered around IDH and 1p/19q diagnostics. This revised classification is reviewed with a focus on adult brain tumors, and includes~~ing~~ a recommendation of genes of which routine testing is clinically useful. Apart from assessment of IDH mutational status incl sequencing of R132H-immunohistochemistry -negative cases and testing for 1p/19q several other markers can be considered for ~~deserve~~ routine testing, including assessment of copy number alterations of chromosome 7 and 10, and of *TERT* promoter, *BRAF* and *H3F3A* mutations. For 'glioblastoma, IDH mutated' the term astrocytoma grade IV c~~s~~hould be considered. It should be considered to treat IDH-wild type grade II and III diffuse glioma with polysomy of chromosome 7 and loss of 10q as glioblastoma. New developments must be more quickly translated into further revised diagnostic categories. Quality control, and rapid integration of molecular findings into the final diagnosis and the communication of the final diagnosis to clinicians require systematic attention.

Keywords: WHO classification, glioma, IDH, 1p/19q codeletion, 7+/10LOH

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'The Genotype trumps the histological phenotype'[1]

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The World Health Organization (WHO) classification of Tumors of the Central nervous System is the standard and universally used diagnostic system for the classification of brain tumors. It was originally built on the morphological appearance of tumor cells and their resemblance to normal brain cells, with a grading system based on the outcome of tumors if left untreated. In recent years however classical histopathology with a limited incorporation of genetic changes was no longer meeting current clinical needs, as illustrated by

- The notorious interobserver variation in the classification and grading of in particular of grade II and III gliomas[2]
- The demonstration that a molecular correlate of oligoastrocytoma does not exist, consistent with large differences in outcome of anaplastic oligoastrocytoma [3-5]
- Molecular reclassification of gliomas containing more prognostic information compared to classical histopathology[6-9]
- ~~Different molecular subclasses of medulloblastoma associated with a different age at presentation and with different outcome~~[10]

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The common denominator in all these observations is the additional information contained in the molecular profile of histologically similar tumors allowing a more accurate classification and better prediction of clinical outcome compared to that according to histology alone. This insight is now reflected in the conceptual change of the 2016 revision of the 'WHO Tumours of the Nervous System' (table 1).[10~~11~~] In an evidence-based manner, key molecular markers such as mutations in *isocitrate dehydrogenase* (*IDH*) gene and 1p/19q status are now central in the description of brain tumors. For clinicians, this revision is timely and reflects the beginning of an era in which molecular diagnostics are integral to the diagnostic classification. This present review focusses on the major changes the WHO

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2016 brings to the glioma classification of the central nervous system tumors (table 1), and discusses which genetic alterations are useful for routine assessment and their implementation in the clinic. [11][12]

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The WHO 2016 classification: from IDH to Not Otherwise Specified (NOS)

For practicing neuro-oncologists, the changes in the classification of the diffuse gliomas are the most relevant as these are by far the most frequent adult primary brain tumors. The above quote 'genotype trumps phenotype' is limited to the context of glioma diagnostics and is based on the assessment of IDH mutations and 1p/19q status in diffuse glioma. A tumor with oligodendroglial morphology, showing an IDH mutation but no 1p/19q loss will be designated astrocytoma, IDH mutated, whereas tumor with features of a glioblastoma but IDH mutated and 1p/19q co-deleted will be designated an anaplastic oligodendroglioma (figure 1a). For diffuse (anaplastic) astrocytoma and glioblastoma without IDH mutations, the term IDH wild type is used (e.g., astrocytoma IDH wild type). If molecular testing for IDH status could not be completed or was inconclusive the term 'Not Otherwise Specified' (NOS) is used (e.g., resulting in 'glioblastoma 'IDH wild type', glioblastoma 'IDH-mutant', and glioblastoma NOS). ~~This use of NOS is however not recommended~~. Except for childhood oligodendroglioma, the diagnosis (anaplastic) oligodendroglioma requires demonstration of both an IDH mutation and combined 1p/19q loss: the current WHO classification does not consider (anaplastic) oligodendroglioma without IDH mutation and 1p/19q co-deletion a distinct tumor entity. That leaves cases with in which the local pathologist finds an (anaplastic) oligodendroglial morphology but in which neither 1p/19q loss nor an IDH mutation is detectable an orphan category, of unknown frequency and clinical significance. The WHO 2016 classification recommends in that situation to consider other diagnoses, and in particular of glioblastoma in case of combined presence of polysomy of chromosome 7 and loss of 10 (see below) but that indeed typical genetic aberration is still not considered diagnostic (see below). One solution would have been to add to the 2016 classification the categories of (anaplastic) oligodendroglioma, IDHwt, as a way out of

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difficult to diagnose cases that undoubtedly will surface. The use of NOS for childhood oligodendroglioma without 1p/19q loss and IDH mutations is confusing, as in adults the use of this term 'NOS' is restricted to those cases where testing was not possible or was non-conclusive. Future updates of the WHO classification may consider how these disparate clinico-pathologic entities may be classified more precisely. Two other mutations have become diagnostic classifiers: 'RELA fusion positive ependymoma', and 'diffuse midline glioma, H3 K27M mutant'. Despite these changes, in general genotyping alone should not be used for glioma diagnostics: alterations must be understood in the context of the findings of a diffuse glial tumor. Nonetheless, in rare cases histopathology may fail to find evidence of tumor, but genetic analysis may reveal typical alterations allowing a classifying diagnosis (figure 2).

Disappearing glioma entities

With this emphasis on 1p/19q and IDH, mixed oligoastrocytoma do not exist in the molecular WHO 2016 classification and what is left are morphological oligoastrocytoma in which the molecular testing was not completed or inconclusive (NOS). Studies have made clear that mixed oligoastrocytoma are usually either IDH mutated, 1p/19q co-deleted, or IDH mutated but 1p/19q intact; at the molecular level truly mixed tumors do not exist (the rare anecdotal reports do not really contradict that).[3, 12, 13] Hence, similar to the classification of oligodendroglioma, it is inconsistent that we have anaplastic astrocytoma IDH wild type, but no (anaplastic) oligoastrocytoma IDHwt as this can potentially be one of the diagnoses rendered (although the text states that in these in anaplastic mixed cases a glioblastoma should be considered no molecular criteria for this have been defined , see below). Another entity that disappeared from the classification is "gliomatosis cerebri". The prior diagnosis of gliomatosis cerebri was based on the radiological appearance of a diffuse tumor involving more than one lobe without histological

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specifications. It has long been recognized that this definition was very subjective, with outcome and sensitivity to treatment again reflecting the molecular background.[13, 14~~14~~, 15~~15~~] In the current classification, widely infiltrating glioma are now designated according to their molecular profile. Whether widely infiltrative phenotypes have specific clinical correlations compared to more localized tumors requires further study. For clinicians, its use may continue to be helpful for some cases as initial radiotherapy may be less attractive. At present this radiological diagnosis is however quite loosely defined.

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Grading: IDH mutant astrocytoma grade IV vs glioblastoma?

The revised WHO 2016 does not address grading at the molecular level. There are several reasons for this. First, in IDH mutated histologically grade II and III tumors the impact of histological grade on survival may be less compared to the impact of grade in tumors of unknown IDH mutational status .[15~~15~~16~~16~~] Clearly though, histopathological characteristics do have an impact on outcome on IDH mutated tumors as well, as grade IV IDH mutated glioblastoma tend to have a worse outcome compared to grade II and III tumors. A re-analysis of The Cancer Genome Atlas (TCGA) confirmed the relevance of grade in all molecular subtypes of diffuse glioma.[16~~16~~17~~17~~] At present, there are however insufficient data on molecular abnormalities within molecularly defined subgroups that allow a robust and reproducible prognostication. Although some lesions indicative of poor prognosis have been identified (e.g., LOH 9p in 1p/19q co-deleted tumors, PI3 kinase mutations in IDH mutated 1p/19q intact tumors), they need validation in larger and independent series .[16-18~~17-19~~] But it appears a missed opportunity that the naming of 'glioblastoma, IDH mutant' has not been further addressed. In the WHO 2016 these tumors continue to be lumped with the variants of glioblastoma, but these tumors are different from a metabolic perspective, occur in younger patients and have a better outcome compared to *IDHwt* glioblastoma. To be consistent, a consideration would have been to label these tumors astrocytoma grade IV IDH mutant

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in order to distinguish them from IDHwt glioblastoma. That would also have put the *IDH* mutation at the heart of the 'astrocytoma' diagnosis, similar to the role of the 1p/19q co-deletion in oligodendroglioma. It would reflect the molecular similarities of these tumors, and the gradual and subjective differences between grade II, III and IV IDH mutated astrocytic tumors.

The genetic identification of glioblastoma in histological grade II and III lesions

The absence of IDH mutations confers a worse prognosis in diffuse grade II and III glioma, but much more can be said about these tumors. Indeed, some I*DHwt* diffuse astrocytoma or the oligoastrocytoma/oligodendroglioma present without histological features of glioblastoma (necrosis, endothelial proliferation) have genetic lesions typical of glioblastoma: gain of chromosome 7, loss of 10q and *TERT* promoter *(TERTp)* mutations. [6, 8, 19~~6, 8, 20~~] Usually these patients are 50 years or older, and they typically have a poor outcome. Some of these cases may be explained by sampling error obtained of ring enhancing lesions with a necrotic center, but others are observed in sometimes large tumors without any enhancement on MR scanning. Although the WHO classification mentions that in 1p/19q intact anaplastic oligodendroglioma and in anaplastic oligoastrocytoma with gain of 7 and loss of 10 a glioblastoma must be considered, these tumors continue to be diagnosed as astrocytoma, IDH-wild type or oligodendroglioma/oligoastrocytoma (figure 1b~~3~~). The same holds true for entities in which only *TERTp*mutations are found without an *IDH* mutations and which usually have a clinical course similar to glioblastoma.[20~~21~~] If it is accepted that "genotype trumps phenotype", then the WHO classification could consider going beyond the *IDHwt* diagnoses, and make a next classifying step in grade II and III tumors with glioblastoma-like molecular characteristics. Clinicians are already becoming inclined to treat these tumors like glioblastoma, indeed one approach could be to call them 'grade III glioblastoma'. Signature GBM molecular alterations should be codified to define these tumors, as clearly other subsets of IDHwt

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LGG's do not have the molecular characteristics of GBM and instead represent other entities on a biologic level.

~~Medulloblastoma~~

~~The other group with major changes are the medulloblastomas. Four different molecular subtypes of medulloblastoma are defined: Wingless-type MMTV integration site family member (Wnt) activated, sonic hedgehog (SHH) activated and TP53 mutant, SHH activated and TP53 wild type and medulloblastoma non-Wnt/non-SHH comprising both group 3 and group 4 medulloblastoma. This classification reflects the revolutionary insights in the molecular classification of medulloblastoma into four subtypes, obtained in large scale collaborations.[10] For medulloblastoma, the fallback position for no molecular diagnostics is the classical histopathological definition (classical, desmoplastic/nodular, extensive nodularity and large cell/anaplastic). The chapter on this entity makes some ambiguity clear: both a classification based on molecular biology and on classical pathology are offered.~~

Ependymoma

New studies on large multicenter datasets on ependymoma have yielded an enormous amount of new biological knowledge.[21, 22] ~~(Pajtler, 2015 2582 /id;~~ These have resulted in proposal for an ependymoma classification in 9 subgroups, or 6 if subependymoma are left out. ~~At the molecular level ependymoma subgroups exists,~~ In supratentorial ependymoma fusion genes involving ~~and a~~ RELA (occurring in up to 88% of childhood supratentorial ependymoma) and YAP1 (10%) have been identified ~~fusion protein has been identified which occurs in up to 70% of childhood supratentorial ependymoma~~ which are absent ~~but not~~ in fossa posterior ependymoma. [21][22] The RELA fusion ependymoma are now part of the WHO 2016 classification, but not the YAP1 fusion ependymoma. Using methylation arrays~~In~~ in fossa posterior ependymoma, two completely different subtypes of fossa posterior ependymoma subtypes can be distinguished: ~~genome wide methylation arrays are able to distinguish between~~ EPN-PFA (high risk for

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progression, median age at diagnosis 3 years but occurring in 11% of adults), -and low risk tumors (good prognosis, EPN-PFB, occurring in 45% of ependymoma patients between 10 and 17% and in most patients over 18 years). Importantly, classification using methylation arrays has more prognostic and diagnostics significance compared to classical histopathology. In fact, the currently available data suggest that analysis with methylation arrays a in a clinically relevant distinction between tumors that post-operatively need further RT because of poor prognosis (EPN-PFA) and favorable prognosis tumors (EPN-PFB) that after extensive resection allow a conservative approach; whereas histopathology does not allow this distinction.[22~~23~~] This is another area where clinical knowledge already deviate from the WHO 2016 diagnostic classification, and with therapeutic implications. , ~~which potentially could be used for clinical decision making on post-operative radiotherapy.~~ [~~23~~] ~~That is however not part of the current classification, and~~ This example further emphasizes the need to continue the refining of molecular diagnostics and its incorporation into the WHO classification, and the need to consider non-mutational diagnostics (here: epigenetics) as clinically relevant classifiers.

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The 7-year cycle of WHO: beyond the realm of pathology

Indeed, the WHO classification of brain tumors is a moving target: as time goes by, novel molecular entities will be defined (and with the observations on 7+/LOH10q tumors, at the time of the WHO 2016 publication the field has moved already).[22~~23~~] The mission of the WHO "blue book" series is to provide a description of neoplastic entities that balances the need for a universally applicable system of classification, while at the same time allows for changes that are warranted based on the evidence from current research. In so doing it acknowledges that while appropriate molecular markers can be critical to classify tumors appropriately, they are not always universally available and in such cases, allowance must be made to ensure and promote, to the extent possible, an accurate classification that can be widely applied. That automatically implies though that this diagnostic standard may not reflect the advance of

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medical care. As long as these new developments have only limited clinical correlates (either in terms of prognosis or treatment options) this will be not be a major issue, but once these findings have clinical implications that perception will rapidly change and friction arises with the clinicians using the diagnostic system for day to day treatment decisions. Another consequence of the rapid genetic developments is that revisions are required more frequently. To that end, the recent iteration of the WHO classification was termed an "update", in accordance with the queue in the WHO blue book series. It is estimated that in several years the time will be appropriate for the formal revision, but facts occurring 'on the ground' may dictate otherwise and require earlier revisions. In addition, it is axiomatic that this diagnostic classification requires more diverse multidisciplinary input, including molecular biologists, clinicians and radiologists, who represent the "end-users" of the classification. A 'worst case scenario' is an 'exit' variant in the field of neuro-oncology: clinicians defining their own classification.

Which genes should be routinely assessed?

For glial tumors, the emphasis in the WHO 2016 classification is on *IDH* and 1p/19q. More frequently mutated genes have however been identified in glioma, like *CIC*, *FUBP* and *ATRX*, many of which appear to be subclonal.[6, 23-26, 24, 27] Others are clearly clonal, like *TERTp* and *TP53*. They currently serve no role in the WHO 2016 classification but they may have some significance though, especially if more advance diagnostics platforms are used that routinely assess a wider spectrum of abnormalities. Incorporating these in routine diagnostics may help to better understand the overall picture, and increase the overall reliability of a molecular diagnosis even if they are not essential for any diagnosis. In contrast, other rarer mutations in *BRAF* and histone genes (*H3F3A*, *HIST1H3B*) indeed identify tumors with specific clinical characteristics. Of these, *H3F3A* K27M mutations have been included in the new WHO classification, with the designation of a new entity, the 'diffuse midline glioma, H3 K27M mutant'. This raises the question

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as to which should be routinely assessed, which are optional but nice to have and which are without clinical relevance.

1p/19q co-deletion.

This is now part of standard diagnostics. 1p/19q loss was first identified in 1994 as the most characteristic genetic lesion in oligodendroglioma, associated with chemotherapy response in 1998, and subsequently assumed to be both prognostic for survival and predictive for benefit from the addition of PCV chemotherapy to radiotherapy.[27-30~~28-31~~] This combined 1p/19q loss is the result of a still poorly understood balanced translocation, in which both the whole p-arm of chromosome 1 and the whole q arm of chromosome are lost ('classical' 1p/19q co-deletion).[31, 32~~32, 33~~] More recent data suggest that typical 1p/19q loss is always associated with IDH mutations.[6, 7, 26, 33~~6, 7, 27, 34~~] Since 1p and 19q loss occasionally occurs in other tumors, the finding of a 1p/19q deletion in the absence of an IDH mutation does not allow the diagnosis of an oligodendroglioma (figure 3~~4~~). In childhood tumors with histopathological appearance of an oligodendroglioma 1p/19q loss is usually absent, but 1p/19q co-deletion is occasionally identified in newly diagnosed oligodendroglial tumors in patients beyond 65 years of age.[34~~35~~]

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IDH mutations

Assessing IDH mutations is now also part of standard diagnostics. Two types of *IDH* mutations are observed in glioma: in the IDH1 and in the IDH2 gene. All mutations in *IDH1* and *IDH2* are somatic, missense, heterozygous, and affect codon 132 (*IDH1*) or codon 172 (*IDH2*). *IDH* mutations are mutually exclusive; 90% of all *IDH* mutations concern the *IDH1* R132H mutation. Studies have shown that *IDH* mutations are early events in gliomagenesis, and remain present at the time of tumor progression.[35, 36~~36,~~]

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[37] *IDH* mutated tumors occur in all grade II-IV diffuse glioma, but are absent in other primary brain tumors. If found in other histological subtypes this is likely to represent a histopathological misclassification. *IDH* mutations are common in adult grade II and III glioma, occurring in 70-80% of cases.[36, 37][37, 38] About 5-10% of glioblastoma show *IDH* mutations, in particular in patients below 50 years of age. In pediatric glioma *IDH* mutations are rare, but have been described in patients as young as 12 years of age.[38][39] *IDH* mutated tumors have an improved outcome compared to non-IDH mutated tumors of similar histopathological grade. *IDH* mutations cause an altered enzyme substrate affinity, leading to increased levels of 2-hydroxyglutarate and lower levels of α-ketoglutarate.[39][40] One of the metabolic alterations that this induces is the development of a global methylation of CpG islands, including the M*GMT* gene promoter. This may explain some of the chemotherapy sensitivity of *IDH* mutated tumors; another explanation is that some of the chemotherapy resistance mechanisms are depending on α-ketoglutarate.[40][41] It has been suggested *IDH* mutations can be used to identify patients that will benefit from adding chemotherapy to radiotherapy, other studies were however not confirming this and identified *MGMT* promoter methylation as the best predictive factor which is however usually present in *IDHmt* tumors.[41, 42][42, 43]

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TP53

TP53 mutations are predominantly observed exon 4-8, and occur in 95% of *IDH* mutated tumors without 1p/19q co-deletion. They do however also occur in other glial tumors, including glioblastoma, in 1p/19q co-deleted tumors (although less frequently), in medulloblastoma and in pediatric glioma. Therefore they lack diagnostic specificity and in glial tumors they are not associated with treatment outcome. There is currently no role for routine testing; if diagnosed they may support the diagnosis of several entities.

Alpha-thalassemia syndrome gene (ATRX)

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Mutations in the ATRX gene occur in 70% of IDH mutated gliomas without 1p/19q co-deletion, the astrocytic type of glial tumor. They are mutually exclusive with *TERT* promoter (*TERTp*) mutations. There are no hot spot regions for *ATRX* mutations, and they can be sub-clonal with different *ATRX* mutations in different parts of the tumor and with different *ATRX* mutations at first diagnosis vs recurrent tumors. If present, they suggest an IDH mutated TP53 mutated astrocytoma. *ATRX* mutations also occur in H3 mutated tumors. *ATRX* mutations can be assessed by immunohistochemistry and by sequencing. Loss of *ATRX* IHC staining in mutated tumors can be a rapid method to detect *ATRX* mutations, and it has been suggested it may obviate the need for 1p/19 testing.[9] While some neuropathologists use ATRX IHC as a criterion to select which gliomas are to be tested for 1p/19q status, further experience is needed to test whether it can substitute for a 1p/19q test, but for now, the WHO 2016 classification explicitly does not accept positive staining for *ATRX* in *IDH* mutated tumors as an alternative to diagnose 1p/19q codeleted *IDH* mutated oligodendroglioma.

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Telomerase Reverse Transcriptase promoter ((TERTp) mutations

Somatic hot spot mutations in the *TERTp* gene occur in *IDHwt* glioblastoma and in 1p/19q co-deleted IDH mutated oligodendroglioma. As a consequence, simply assessing both *TERTp* and *IDH* mutational status already results in a very powerful prognostic glioma classification.[7, 20, 43~~7, 21, 44~~] In some tumors only *TERTp* mutations are found, without other typical glioma alterations; these patients tend to have a poor outcome. *TERTp* mutations are mutually exclusive with ATRX mutations. Interestingly, patients with grade II and III *IDHwt* tumors but without a *TERTp* mutations appear to have a better prognosis compared to patients with *TERTp* mutations. Typically, these studies have been lacking the assessment of chromosome 7 and 10q, which most likely would have identified a glioblastoma like chromosomal loss pattern in many of the IDHwt/*TERTp* mutated tumors. More clinical outcome data on these tumors are

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urgently needed. Assessment of *TERTp* mutational status can be useful for *IDHwt* diffuse glioma, they are however not specific for glioma and for example occur also in medulloblastoma.

The gain of 7 and loss of 10q genotype

The combination of tri/polysomy of chromosome 7 and LOH of 10q is a characteristic combination found in many glioblastoma and probably represents an early event in these tumors.[19, 44~~20, 45~~] Usually *TERTp* mutations are present, and in 40-50% of cases *EGFR* amplification usually with *EGFR* mutations, including *EGFRvIII* mutations in 20%. Many *IDHwt* astrocytoma and anaplastic astrocytoma (especially in patients over 45 years of age) show this 7+/10q- pattern, and typically have a clinically aggressive course (figure 1b~~3~~).[6, 45~~6~~] Testing for this combination in patients over 45-50 years of age with grade II of III *IDHwt* tumors may give positive indications for a poor prognosis. The WHO classification strongly suggests the diagnosis glioblastoma should be considered in 7+/10q- anaplastic oligodendroglioma and anaplastic oligoastrocytoma, but these abnormalities do not qualify for the diagnosis glioblastoma ion the current classification. The available clinical data support that despite these being histologically grade II or III tumors, that these tumors should be treated as glioblastoma and many clinicians with routine access to diagnostics of 7 and 10q do so.

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Epidermal Growth Factor Receptor (EGFR) amplification and mutations

EGFR amplification occurs in 40-50% of all glioblastoma, and is usually associated with EGFR mutations and trisomy/polysomy of chromosome 7.[46] Most *EGFR* amplified tumors also show *EGFR* mutations affecting the extracellular domain of the receptor, the most frequent being the *EGFRvIII* mutation. There is currently no drug that specifically or effectively exploits any of these mutations, although several trails on novel agents are ongoing. As a consequence, from both a therapeutic and a diagnostic aspect the routine assessing of *EGFR* amplification or *EGFR* mutations is currently not useful. The presence of EGFR amplification is indeed highly specific: if found it is diagnostic at the molecular level of a glioblastoma,

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but it lacks sensitivity: assays for EGFR amplification will be negative in 50% of the glioblastoma cases. Outcome of *EGFR* amplified or *EGFRvIII* mutated tumors is not different from other glioblastoma.[47] Currently, for glioblastoma diagnostics assessing both chromosome 7 and 10q or *TERTp* mutations is more informative than assessing EGFR amplification status.

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Phosphatase and Tensin Homolog (PTEN)

PTEN mutations occur in 20-30% of glioblastoma, and are as a rule accompanied by LOH10q. When both are present this results in bi-allelic PTEN inactivation. They may also occur at low frequency in other gliomas with unclear clinical significance, and in other tumors (medulloblastoma). Thus, it has low diagnostic value and no therapeutic consequences. Routine assessment of PTEN mutations is clinically not indicated.

BRAF-KIAA fusion genes and BRAF mutations in glial tumors

Abnormalities in the BRAF gene are characteristic of several subgroup of gliomas. Pilocytic astrocytoma (PA) in the fossa posterior typically have a tandem duplication at 7q34 resulting in a transforming fusion gene between *KIAA1549* and *BRAF* (*BRAF* duplication or *BRAF-KIAA1549* fusion gene), but not the *BRAFv600* mutation. *BRAF-KIAA* fusion genes are also frequent in non-NF1 optic nerve glioma (73%).[48] *BRAF-KIAA*1549 fusion are age specific, they are rare in PA patients over 40 years of age (7%). BRAFv600 mutations are mutually exclusive with the BRAF-KIAA549 fusion gene, these are observed in , 33% of the non-posterior fossa PA. They are also relatively common in pleomorphic xantroastrocytoma (PXA; 43-66%), anaplastic PXA (65%) and ganglioglioma (18-43%) especially if located in the brain stem[49-52]; they are rare in adult glioma (glioblastoma: 2%, adult low grade glioma: 0-3%).[49] They are also frequent in the proposed novel (but rare) WHO entity of epitheloid glioblastoma, although their distinction from anaplastic PXA is unclear.[53] A study on pediatric diencephalic low grade glioma reported frequent *BRAFv600* mutated non-PA in this region, with imaging characteristics of vivid enhancement and

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multiloculated or multinodular appearance and/or infiltrative growth on T2 weighted images (figure 2).[54] Field Code Changed

A Canadian series observed *BRAF* fusion positivity in unilateral thalamic low grade tumors.[55] Since *BRAF* Field Code Changed mutated tumors may be treated with targeted agents aiming at the *BRAFv600* mutations, either alone or in combination with a MERK pathway inhibitor, the finding of this abnormality may have therapeutic implications. Responses to these agents have been described, and this appears a very promising avenue of research.[56] *BRAF* mutations and the *BRAF-KIAA* fusion have not been incorporated into the current Field Code Changed diagnostic classification; the diagnosis of pilocytic astrocytoma remains a morphological definition. Routine testing must be considered in relevant cases. Future research should focus on establishing to what extent these tumors share the same background, and to what extent other abnormalities in the *RAS/RAF* pathway may have a similar phenotypic effect. More rare genetic lesions in pilocytic astrocytoma include *NF1*, *KRAS* and *RAS* mutations and *FGFR1* and other *BRAF* fusions.[57] Field Code Changed

Histone H3F3A and HIST1H3B mutations

The WHO 2016 has accepted the 'diffuse midline glioma, H3 K27M mutant' as diagnostic entity, occurring predominantly in childhood and adolescent brain tumor patients. The mutation is part of a larger family of histone mutations with similar clinical presentation. Pediatric and young adult glioma frequently show mutations in genes encoding H3 variants, which through histone modification alter gene expression.[58] Driver mutations occur in the *H3F3A gene* (positions K27 and G34) encoding histone H3.3 Field Code Changed genes, and in *HIST1H3B* histone H3.1 gene (K27 position). K27 mutated tumors typically arise in the brainstem and midline structures such as thalamus and cerebellum, mostly in children and young adults. Thus, diffuse intrinsic pontine glioma (DIPG) frequently harbor K27M mutations in histone H3.3 genes as well as in H3.1 genes.[59] Childhood and young adult supratentorial glioma may show mutations in histone Field Code Changed H3.3, with K27M mutations occurring in midline tumors. In contrast, pG34R/V histone H3.3 mutations are restricted to pediatric and young adult HGGs of the cerebral cortex, and are almost invariably

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associated with *ATRX* and *TP53* mutations.[59, 60] K27 mutations are associated with a poor outcome; G34 mutations appear to have better survival. Intrinsic pontine glioma harboring a K27M mutation in H3.3 are less responsive to radiotherapy, with earlier relapses and more metastatic recurrences than those in H3.1.[60] Although the K27M mutation was frequently observed in adult brainstem and thalamic gliomas, this mutation tended to be associated with a poorer prognosis in brainstem gliomas but not in thalamic gliomas.[61] The presence of the H3F3A K27M mutation is associated with mutations in *TP53*.[59, 62] An antibody against the K27M allele may prove useful to facilitates detection of this mutation.[63] The role of other mutations (e.g., ACVR1) in DIPG remain to be elucidated. Testing for *H3F3A* mutations is insightful in pediatric and young adult cases with midline tumors.

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Challenges: platforms and tests to be used

The revised WHO 2016 criteria do not make recommendations how to assess molecular alterations, which is wise in view of the rapidly changing landscape of molecular diagnostics and testing platforms. With next generation sequencing techniques (NGS) becoming rapidly more affordable, NGS panels tailored for glioma diagnostics are increasingly being used for routine diagnostics including assessment of copy number alterations.[64] Although there is clearly an advantage of the assessment of more than only *IDH* mutation and 1p/19q status, the routine use of screening for the 50 most frequent cancer genes or whole exome in glioma is without clinically proven benefit. Outside the identification of molecular glioblastoma with WHO grade II or III histology, no proven therapeutic decisions can be taken based on these profiles, with *BRA*F mutant tumors as the most promising exception as they allow patients to be selected for clinical trials.[56] Previous studies have shown the clinical usefulness of gene expression analysis and genome wide methylation analysis. In particular, the latter approach has been shown to be very informative, allowing the classification of tumors without knowledge of specific

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mutations. This classification system is based on the assumption that the methylation pattern of a tumor is the consequence of both the lineage of the cell the tumor arises from, and tumor specific DNA characteristics. For brain tumors, a relevant aspect here is that the analysis of methylation status simultaneously allows the assessment of *MGMT* status, which may well be the single most powerful determinant of benefit from alkylating agent chemotherapy.[42, 45~~43, 65~~]

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Assessment of molecular characteristics in every day practice and pitfalls

Testing for *IDH* is part of routine diagnostics, but it seems reasonable to limit routine testing to an age range of 15 to 55 - 60 years, for example, and test beyond that only on clinical indications (e.g. in all adult grade II and III glioma, in the presence of oligodendroglial features, and in case of a hemispheric astrocytoma in a 13 year old). For the *R132H* mutation a very reliable immunohistochemistry (IHC) assay is available, but this represents only 90% of all *IDH* mutations. As a consequence, IHC has at best a 90% sensitivity, implying that in case of IHC negativity this must be followed by sequencing for both *IDH1* and *IDH2* mutations (figure 4~~5~~). IHC can be used as a first screen, but not as a tool to rule out *IDH* mutations. Testing for 1p/19q status and *IDH* mutations should be performed in all patients presenting with possible oligodendroglial tumors. Testing for 1p/9q status should use an assay that allows assessment of loss of the entire 1p and 19q arm. Fluorescence in situ hybridization (FISH) for 1p using a probe for 1p36.6 region is less specific as it may suggest loss ~~be positive~~ in tumors with partial 1p deletion only, limited to the tip of chromosome 1p.[65, 66~~66, 67~~] This part can be lost without loss of the rest of chromosome 1p, which in combination with 19q loss has been observed in glioblastoma. If copy number alterations (CNA) are considered to be relevant this should be assessed with other techniques. Both 1p/19q co-deletions and IDH mutations are early events in gliomagenesis, and their presence or absence are unlikely to change over time.[67, 68~~68, 69~~] Therefore, retesting of 1p/19q and *IDH* status at the time of a re-resection in tumors with already known status is of limited use, unless a significant clinical change

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occurred indicating a second tumor. Incorporation of assessment of *TERTp* mutations into routine diagnostics of gliomas has been suggested.[20,21] In the absence of 1p/19q loss, diffuse gliomas with *TERTp* mutations tend to have a poor outcome reminiscent of glioblastoma.[7, 43,44] Although some studies on targeted mutation assessment have shown that in some tumors only *TERTp* mutations were observed, this deserves further clinical study since in most of these series tumors were not tested for CNA of 7 and 10q.

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Reporting

Centers must develop automated workflows that incorporate molecular testing in their routine procedures, including the incorporation of the molecular diagnostics in the final pathology result. It is important for the reporting of the diagnosis to be standardized and made available for capture in the national cancer registry databases, so the incidence of the specific entities based on molecular features can be reported. Since the turnaround time for histopathology is shorter than for molecular diagnostics, ensuring accurate and timely feedback on molecular findings in patients in whom a histopathological diagnosis has already been established is important. It is recommended to routinely include (MR) imaging characteristics in the final diagnostic considerations: MRI images should be consistent with the pathological diagnosis and if not this should give rise to additional scrutiny. The interpretation of molecular findings depends on the context: if the tumor is unlikely to be a diffuse glioma the molecular findings may not contribute except when an diagnostic mutation is found with supportive MR and clinical findings.

Clinical studies

With the new classification, the clinical data from past trials without molecular analysis have become outdated. Since the first results of the trials on anaplastic oligodendroglioma, follow-up trials in the newly diagnosed setting (CODEL, CATNON) but also in recurrent disease (TAVAREC) enrolled patients

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based on their 1p/19q status because of the difference in prognosis, and many prospective trials reported retrospectively on the molecular status (table 3). New studies should now distinguish between IDH mutant tumors, IDH mutant and 1p/19q co-deleted, and IDH wild type diffuse gliomas. This complicates matters. As an example, the presence of IDH mutations also identifies a more favorable subgroup of glioblastoma which may also hold true at the time of recurrence.[69,70] This questions whether these tumors should be enrolled in trials on recurrent glioblastomas and whether it should be routinely tested for. On the other hand, the still modest survival of IDH mutated glioblastoma also argues against enrolling these tumors in trials aiming at IDH mutated diffuse grade II and III glioma although the limited difference in outcome between grade II and III IDH mutated tumors provides a rational for combining these grades.[15,16] Today's changes emphasize that all trials should collect tissue samples as part of the study design. Analysis of existing datasets may help to improve our understanding of the outcome of these subsets of patients.

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Quality control

~~In the first discussions on the incorporation of the molecular findings in the diagnosis of gliomas a multilayer diagnostic approach was proposed.[71] This concept has been abandoned in the final WHO 2016 revision, which unifies the diagnosis with the molecular diagnostics as the essential part of glioma classification. Some have proposed that the addition of the histopathological diagnosis may still be useful as it may contribute to the granularity of the diagnosis.~~ The interobserver variation in the histopathological classification of glioma is well known, but early experiences with inter-laboratory tests on diagnostic molecular assays on the same set of tumors revealed that differences between laboratories may exist as well.[70, 71,72,73] Proper quality control is critical now that diagnostics and clinical decisions are based on molecular testing. Laboratories need to certify and validate their testing

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procedures with appropriate controls. This is not exciting work and requires significant efforts, but is absolutely essential for reliable diagnostics.

Conclusion.

The new WHO 2016 classification for brain tumors brings molecular diagnostics to the center of glioma ~~and medulloblastoma~~ classification. This revised classification will improve treatment selection of brain tumor patients and clinical trial design. This will not be the last revision of this classification as new molecular insights into brain tumors will further refine the classification of brain tumors. Further refinements already seem indicated, e.g., in the IDH wild type categories of grade II and III glioma as these represent in many cases –especially in patients over 50 years of age- glioblastoma -like lesions with 7+/10q LOH. Further analysis of *TERTp* mutational status in non-glioblastoma 1p/19q intact tumors will be needed to better understand the prognostic role of that mutation in diffuse glioma. More responsiveness to the rapidly changing and multidisciplinary field of neuro-oncology will be crucial to maintain a well-accepted WHO classification of brain tumors. For this, a more transparent and multidisciplinary process of change of these pivotal criteria will be needed.

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Figures

Figure 1 Glioblastoma diagnostics anno 2016. 1a. T1 weighted MR images of a 40 year old male with a short history headache and difficulty walking . At histopathology a glioblastoma was diagnosed, targeted sequencing showed ~~b~~ an *IDH2* mutation, combined 1p/19q loss and deletion of chromosome 9 consistent with the diagnosis of an anaplastic oligodendroglioma; 1b. T1 weighted contrast enhanced MR image of a 50 year old female who developed over months progressive memory and behavioral complaints. No contrast enhancement was present, at biopsy histopathology showed a grade II astrocytoma, Next Generation Sequencing failed to show an *IDH* mutation but instead documented gain of chromosome 7, loss of 10q, and mutations in the EGFR and PTEN gene consistent with a glioblastoma.~~.~~

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Figure 2. T2 weighted images of a 25 year old male who underwent two biopsies for a mesencephalic lesion, on both occasions histopathological examination failed to show clear evidence of tumor. On mutational analysis, a *BRAF* mutation was found (c.1795_1797dupACA;p.T599dup)

Figure 3. ~~T1 contrast enhanced MR images of a ring right frontal enhancing lesion tumor histopathologically classified as glioblastoma, at molecular examination 1p/19q co-deletion was found but with neither an *IDH* nor a *TERTp* mutation,~~

~~Figure 4. T1 weighted contrast enhanced MR image of a 50 year old female who developed over months progressive memory and behavioral complaints. No contrast enhancement was present, at biopsy histopathology showed a grade II astrocytoma, Next Generation Sequencing failed to show an *IDH* mutation but instead documented gain of chromosome 7, loss of 10q, and mutations in the EGFR and PTEN gene consistent with a glioblastoma.~~

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Figure 3. T1 contrast enhanced MR images of a ring right frontal enhancing lesion tumor histopathologically classified as glioblastoma, at molecular examination 1p/19q co-deletion was found but with neither an *IDH* nor a *TERTp* mutation.

Figure ~~5~~4. T2 weighted MR images of a r~~R~~ight frontal low grade astrocytoma in a 20 year old, diagnosed as *IDH wt* because of negative immunohistochemistry. After referral to a tertiary care center sequencing demonstrated an *IDH* c.394C>T; p.R132C mutation in combination with a *TP53* and an *ATRX* mutation.

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Review. A clinical perspective on the 2016 WHO brain tumor classification and routine molecular diagnostics.

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Abstract

The 2007 WHO classification of brain tumors did not use molecular abnormalities as diagnostic criteria. Studies have shown that genotyping allow a better prognostic classification of diffuse glioma with improved treatment selection. This has resulted in a major revision of the WHO classification, which is now for adult diffuse glioma centered around IDH and 1p/19q diagnostics. This revised classification is reviewed with a focus on adult brain tumors, and includes a recommendation of genes of which routine testing is clinically useful. Apart from assessment of IDH mutational status incl sequencing of R132H-immunohistochemistry negative cases and testing for 1p/19q several other markers can be considered for routine testing, including assessment of copy number alterations of chromosome 7 and 10, and of *TERT* promoter, *BRAF* and *H3F3A* mutations. For 'glioblastoma, IDH mutated' the term astrocytoma grade IV could be considered. It should be considered to treat IDH-wild type grade II and III diffuse glioma with polysomy of chromosome 7 and loss of 10q as glioblastoma. New developments must be more quickly translated into further revised diagnostic categories. Quality control, and rapid integration of molecular findings into the final diagnosis and the communication of the final diagnosis to clinicians require systematic attention.

Keywords: WHO classification, glioma, IDH, 1p/19q codeletion, 7+/10LOH

'The Genotype trumps the histological phenotype'[1]

The World Health Organization (WHO) classification of Tumors of the Central nervous System is the standard and universally used diagnostic system for the classification of brain tumors. It was originally built on the morphological appearance of tumor cells and their resemblance to normal brain cells, with a grading system based on the outcome of tumors if left untreated. In recent years however classical histopathology with a limited incorporation of genetic changes was no longer meeting current clinical needs, as illustrated by

- The notorious interobserver variation in the classification and grading of in particular of grade II and III gliomas[2]
- The demonstration that a molecular correlate of oligoastrocytoma does not exist, consistent with large differences in outcome of anaplastic oligoastrocytoma [3-5]
- Molecular reclassification of gliomas containing more prognostic information compared to classical histopathology[6-9]

The common denominator in all these observations is the additional information contained in the molecular profile of histologically similar tumors allowing a more accurate classification and better prediction of clinical outcome compared to that according to histology alone. This insight is now reflected in the conceptual change of the 2016 revision of the 'WHO Tumours of the Nervous System' (table 1).[10] In an evidence-based manner, key molecular markers such as mutations in *isocitrate dehydrogenase* (*IDH*) gene and 1p/19q status are now central in the description of brain tumors. For clinicians, this revision is timely and reflects the beginning of an era in which molecular diagnostics are integral to the diagnostic classification. This present review focusses on the major changes the WHO 2016 brings to the glioma classification of the central nervous system tumors (table 1), and discusses which genetic alterations are useful for routine assessment and their implementation in the clinic. [11]

The WHO 2016 classification: from IDH to Not Otherwise Specified (NOS)

For practicing neuro-oncologists, the changes in the classification of the diffuse gliomas are the most relevant as these are by far the most frequent adult primary brain tumors. The above quote 'genotype trumps phenotype' is limited to the context of glioma diagnostics and is based on the assessment of IDH mutations and 1p/19q status in diffuse glioma. A tumor with oligodendroglial morphology, showing an IDH mutation but no 1p/19q loss will be designated astrocytoma, IDH mutated, whereas tumor with features of a glioblastoma but IDH mutated and 1p/19q co-deleted will be designated an anaplastic oligodendroglioma (figure 1a). For diffuse (anaplastic) astrocytoma and glioblastoma without IDH mutations, the term IDH wild type is used (e.g., astrocytoma IDH wild type). If molecular testing for IDH status could not be completed or was inconclusive the term 'Not Otherwise Specified' (NOS) is used (e.g., resulting in 'glioblastoma 'IDH wild type', glioblastoma 'IDH-mutant', and glioblastoma NOS). . Except for childhood oligodendroglioma, the diagnosis (anaplastic) oligodendroglioma requires demonstration of both an IDH mutation and combined 1p/19q loss: the current WHO classification does not consider (anaplastic) oligodendroglioma without IDH mutation and 1p/19q co-deletion a distinct tumor entity. That leaves cases with in which the local pathologist finds an (anaplastic) oligodendroglial morphology but in which neither 1p/19q loss nor an IDH mutation is detectable an orphan category, of unknown frequency and clinical significance. The WHO 2016 classification recommends in that situation to consider other diagnoses, and in particular of glioblastoma in case of combined presence of polysomy of chromosome 7 and loss of 10 (see below) but that indeed typical genetic aberration is still not considered diagnostic (see below). One solution would have been to add to the 2016 classification the categories of (anaplastic) oligodendroglioma, IDHwt, as a way out of difficult to diagnose cases that undoubtedly will surface. The use of NOS for childhood oligodendroglioma without 1p/19q loss and IDH mutations is confusing, as in adults the use of this term 'NOS' is restricted to those cases where testing was not possible or was non-conclusive. Future updates of the WHO classification may consider how

these disparate clinico-pathologic entities may be classified more precisely. Two other mutations have become diagnostic classifiers: 'RELA fusion positive ependymoma', and 'diffuse midline glioma, H3 K27M mutant'. Despite these changes, in general genotyping alone should not be used for glioma diagnostics: alterations must be understood in the context of the findings of a diffuse glial tumor. Nonetheless, in rare cases histopathology may fail to find evidence of tumor, but genetic analysis may reveal typical alterations allowing a classifying diagnosis (figure 2).

Disappearing glioma entities

With this emphasis on 1p/19q and IDH, mixed oligoastrocytoma do not exist in the molecular WHO 2016 classification and what is left are morphological oligoastrocytoma in which the molecular testing was not completed or inconclusive (NOS). Studies have made clear that mixed oligoastrocytoma are usually either IDH mutated, 1p/19q co-deleted, or IDH mutated but 1p/19q intact; at the molecular level truly mixed tumors do not exist (the rare anecdotal reports do not really contradict that).[3, 12] Hence, similar to the classification of oligodendroglioma, it is inconsistent that we have anaplastic astrocytoma IDH wild type, but no (anaplastic) oligoastrocytoma IDHwt as this can potentially be one of the diagnoses rendered (although the text states that in these in anaplastic mixed cases a glioblastoma should be considered no molecular criteria for this have been defined , see below). Another entity that disappeared from the classification is "gliomatosis cerebri". The prior diagnosis of gliomatosis cerebri was based on the radiological appearance of a diffuse tumor involving more than one lobe without histological specifications. It has long been recognized that this definition was very subjective, with outcome and sensitivity to treatment again reflecting the molecular background.[13, 14] In the current classification, widely infiltrating glioma are now designated according to their molecular profile. Whether widely infiltrative phenotypes have specific clinical correlations compared to more localized tumors requires

further study. For clinicians, its use may continue to be helpful for some cases as initial radiotherapy may be less attractive. At present this radiological diagnosis is however quite loosely defined.

Grading: IDH mutant astrocytoma grade IV vs glioblastoma?

The revised WHO 2016 does not address grading at the molecular level. There are several reasons for this. First, in IDH mutated histologically grade II and III tumors the impact of histological grade on survival may be less compared to the impact of grade in tumors of unknown IDH mutational status .[15] Clearly though, histopathological characteristics do have an impact on outcome on IDH mutated tumors as well, as grade IV IDH mutated glioblastoma tend to have a worse outcome compared to grade II and III tumors. A re-analysis of The Cancer Genome Atlas (TCGA) confirmed the relevance of grade in all molecular subtypes of diffuse glioma.[16] At present, there are however insufficient data on molecular abnormalities within molecularly defined subgroups that allow a robust and reproducible prognostication. Although some lesions indicative of poor prognosis have been identified (e.g., LOH 9p in 1p/19q co-deleted tumors, PI3 kinase mutations in IDH mutated 1p/19q intact tumors), they need validation in larger and independent series .[16-18] But it appears a missed opportunity that the naming of 'glioblastoma, IDH mutant' has not been further addressed. In the WHO 2016 these tumors continue to be lumped with the variants of glioblastoma, but these tumors are different from a metabolic perspective, occur in younger patients and have a better outcome compared to *IDHwt* glioblastoma. To be consistent, a consideration would have been to label these tumors astrocytoma grade IV IDH mutant in order to distinguish them from IDHwt glioblastoma. That would also have put the *IDH* mutation at the heart of the 'astrocytoma' diagnosis, similar to the role of the 1p/19q co-deletion in oligodendroglioma. It would reflect the molecular similarities of these tumors, and the gradual and subjective differences between grade II, III and IV IDH mutated astrocytic tumors.

The genetic identification of glioblastoma in histological grade II and III lesions

The absence of IDH mutations confers a worse prognosis in diffuse grade II and III glioma, but much more can be said about these tumors. Indeed, some I*DHw*t diffuse astrocytoma or the oligoastrocytoma/oligodendroglioma present without histological features of glioblastoma (necrosis, endothelial proliferation) have genetic lesions typical of glioblastoma: gain of chromosome 7, loss of 10q and *TERT* promoter *(TERTp)* mutations. [6, 8, 19] Usually these patients are 50 years or older, and they typically have a poor outcome. Some of these cases may be explained by sampling error obtained of ring enhancing lesions with a necrotic center, but others are observed in sometimes large tumors without any enhancement on MR scanning. Although the WHO classification mentions that in 1p/19q intact anaplastic oligodendroglioma and in anaplastic oligoastrocytoma with gain of 7 and loss of 10 a glioblastoma must be considered, these tumors continue to be diagnosed as astrocytoma, IDH-wild type or oligodendroglioma/oligoastrocytoma (figure 1b). The same holds true for entities in which only *TERTp* mutations are found without an *IDH* mutations and which usually have a clinical course similar to glioblastoma.[20] If it is accepted that "genotype trumps phenotype", then the WHO classification could consider going beyond the *IDHwt* diagnoses, and make a next classifying step in grade II and III tumors with glioblastoma-like molecular characteristics. Clinicians are already becoming inclined to treat these tumors like glioblastoma, indeed one approach could be to call them 'grade III glioblastoma'. Signature GBM molecular alterations should be codified to define these tumors, as clearly other subsets of IDHwt LGG's do not have the molecular characteristics of GBM and instead represent other entities on a biologic level.

Ependymoma

New studies on large multicenter datasets on ependymoma have yielded an enormous amount of new biological knowledge.[21, 22] These have resulted in proposal for an ependymoma classification in 9 subgroups, or 6 if subependymoma are left out. In supratentorial ependymoma fusion genes involving RELA (occurring in up to 88% of childhood supratentorial ependymoma) and YAP1 (10%) have been identified which are absent in fossa posterior ependymoma. [21] The RELA fusion ependymoma are now part of the WHO 2016 classification, but not the YAP1 fusion ependymoma. Using methylation arrays in fossa posterior ependymoma, two completely different subtypes of fossa posterior ependymoma subtypes can be distinguished: EPN-PFA (high risk for progression, median age at diagnosis 3 years but occurring in 11% of adults), and low risk tumors (good prognosis, EPN-PFB, occurring in 45% of ependymoma patients between 10 and 17% and in most patients over 18 years). Importantly, classification using methylation arrays has more prognostic and diagnostics significance compared to classical histopathology. In fact, the currently available data suggest that analysis with methylation arrays a in a clinically relevant distinction between tumors that post-operatively need further RT because of poor prognosis (EPN-PFA) and favorable prognosis tumors (EPN-PFB) that after extensive resection allow a conservative approach; whereas histopathology does not allow this distinction. [22] This is another area where clinical knowledge already deviate from the WHO 2016 diagnostic classification, and with therapeutic implications. , This example further emphasizes the need to continue the refining of molecular diagnostics and its incorporation into the WHO classification, and the need to consider non-mutational diagnostics (here: epigenetics) as clinically relevant classifiers.

The 7-year cycle of WHO: beyond the realm of pathology

Indeed, the WHO classification of brain tumors is a moving target: as time goes by, novel molecular entities will be defined (and with the observations on 7+/LOH10q tumors, at the time of the WHO 2016 publication the field has moved already).[22] The mission of the WHO “blue book” series is to provide a

description of neoplastic entities that balances the need for a universally applicable system of classification, while at the same time allows for changes that are warranted based on the evidence from current research. In so doing it acknowledges that while appropriate molecular markers can be critical to classify tumors appropriately, they are not always universally available and in such cases, allowance must be made to ensure and promote, to the extent possible, an accurate classification that can be widely applied. That automatically implies though that this diagnostic standard may not reflect the advance of medical care. As long as these new developments have only limited clinical correlates (either in terms of prognosis or treatment options) this will be not be a major issue, but once these findings have clinical implications that perception will rapidly change and friction arises with the clinicians using the diagnostic system for day to day treatment decisions. Another consequence of the rapid genetic developments is that revisions are required more frequently. To that end, the recent iteration of the WHO classification was termed an "update", in accordance with the queue in the WHO blue book series. It is estimated that in several years the time will be appropriate for the formal revision, but facts occurring 'on the ground' may dictate otherwise and require earlier revisions. In addition, it is axiomatic that this diagnostic classification requires more diverse multidisciplinary input, including molecular biologists, clinicians and radiologists, who represent the "end-users" of the classification. A 'worst case scenario' is an 'exit' variant in the field of neuro-oncology: clinicians defining their own classification.

Which genes should be routinely assessed?

For glial tumors, the emphasis in the WHO 2016 classification is on *IDH* and 1p/19q. More frequently mutated genes have however been identified in glioma, like *CIC*, *FUBP* and *ATRX*, many of which appear to be subclonal.[6, 23-26] Others are clearly clonal, like *TERTp* and *TP53*. They currently serve no role in the WHO 2016 classification but they may have some significance though, especially if more advance

diagnostics platforms are used that routinely assess a wider spectrum of abnormalities. Incorporating these in routine diagnostics may help to better understand the overall picture, and increase the overall reliability of a molecular diagnosis even if they are not essential for any diagnosis. In contrast, other rarer mutations in *BRAF* and histone genes (*H3F3A*, *HIST1H3B*) indeed identify tumors with specific clinical characteristics. Of these, *H3F3A* K27M mutations have been included in the new WHO classification, with the designation of a new entity, the 'diffuse midline glioma, H3 K27M mutant'. This raises the question as to which should be routinely assessed, which are optional but nice to have and which are without clinical relevance.

1p/19q co-deletion.

This is now part of standard diagnostics. 1p/19q loss was first identified in 1994 as the most characteristic genetic lesion in oligodendroglioma, associated with chemotherapy response in 1998, and subsequently assumed to be both prognostic for survival and predictive for benefit from the addition of PCV chemotherapy to radiotherapy.[27-30] This combined 1p/19q loss is the result of a still poorly understood balanced translocation, in which both the whole p-arm of chromosome 1 and the whole q arm of chromosome are lost ('classical' 1p/19q co-deletion).[31, 32] More recent data suggest that typical 1p/19q loss is always associated with IDH mutations.[6, 7, 26, 33] Since 1p and 19q loss occasionally occurs in other tumors, the finding of a 1p/19q deletion in the absence of an IDH mutation does not allow the diagnosis of an oligodendroglioma (figure 3). In childhood tumors with histopathological appearance of an oligodendroglioma 1p/19q loss is usually absent, but 1p/19q co-deletion is occasionally identified in newly diagnosed oligodendroglial tumors in patients beyond 65 years of age.[34]

IDH mutations

Assessing IDH mutations is now also part of standard diagnostics. Two types of *IDH* mutations are observed in glioma: in the IDH1 and in the IDH2 gene. All mutations in *IDH1* and *IDH2* are somatic, missense, heterozygous, and affect codon 132 (*IDH1*) or codon 172 (*IDH2*). *IDH* mutations are mutually exclusive; 90% of all *IDH* mutations concern the *IDH1* R132H mutation. Studies have shown that *IDH* mutations are early events in gliomagenesis, and remain present at the time of tumor progression.[35, 36] *IDH* mutated tumors occur in all grade II-IV diffuse glioma, but are absent in other primary brain tumors. If found in other histological subtypes this is likely to represent a histopathological misclassification. *IDH* mutations are common in adult grade II and III glioma, occurring in 70-80% of cases.[36, 37] About 5-10% of glioblastoma show *IDH* mutations, in particular in patients below 50 years of age. In pediatric glioma *IDH* mutations are rare, but have been described in patients as young as 12 years of age.[38] *IDH* mutated tumors have an improved outcome compared to non-IDH mutated tumors of similar histopathological grade. *IDH* mutations cause an altered enzyme substrate affinity, leading to increased levels of 2-hydroxyglutarate and lower levels of α-ketoglutarate.[39] One of the metabolic alterations that this induces is the development of a global methylation of CpG islands, including the M*GMT* gene promoter. This may explain some of the chemotherapy sensitivity of *IDH* mutated tumors; another explanation is that some of the chemotherapy resistance mechanisms are depending on α-ketoglutarate.[40] It has been suggested *IDH* mutations can be used to identify patients that will benefit from adding chemotherapy to radiotherapy, other studies were however not confirming this and identified *MGMT* promoter methylation as the best predictive factor which is however usually present in *IDHmt* tumors.[41, 42]

TP53

TP53 mutations are predominantly observed exon 4-8, and occur in 95% of *IDH* mutated tumors without 1p/19q co-deletion. They do however also occur in other glial tumors, including glioblastoma, in 1p/19q co-deleted tumors (although less frequently), in medulloblastoma and in pediatric glioma. Therefore

they lack diagnostic specificity and in glial tumors they are not associated with treatment outcome. There is currently no role for routine testing; if diagnosed they may support the diagnosis of several entities.

Alpha-thalassemia syndrome gene (ATRX)

Mutations in the ATRX gene occur in 70% of IDH mutated gliomas without 1p/19q co-deletion, the astrocytic type of glial tumor. They are mutually exclusive with *TERT* promoter (*TERTp*) mutations. There are no hot spot regions for *ATRX* mutations, and they can be sub-clonal with different *ATRX* mutations in different parts of the tumor and with different *ATRX* mutations at first diagnosis vs recurrent tumors. If present, they suggest an IDH mutated TP53 mutated astrocytoma. *ATRX* mutations also occur in H3 mutated tumors. *ATRX* mutations can be assessed by immunohistochemistry and by sequencing. Loss of *ATRX* IHC staining in mutated tumors can be a rapid method to detect *ATRX* mutations, and it has been suggested it may obviate the need for 1p/19 testing.[9] While some neuropathologists use ATRX IHC as a criterion to select which gliomas are to be tested for 1p/19q status, further experience is needed to test whether it can substitute for a 1p/19q test, but for now, the WHO 2016 classification explicitly does not accept positive staining for *ATRX* in *IDH* mutated tumors as an alternative to diagnose 1p/19q codeleted *IDH* mutated oligodendroglioma.

Telomerase Reverse Transcriptase promoter ((TERTp) mutations

Somatic hot spot mutations in the *TERTp* gene occur in *IDHwt* glioblastoma and in 1p/19q co-deleted IDH mutated oligodendroglioma. As a consequence, simply assessing both *TERTp* and *IDH* mutational status already results in a very powerful prognostic glioma classification.[7, 20, 43] In some tumors only *TERTp* mutations are found, without other typical glioma alterations; these patients tend to have a poor

outcome. *TERTp* mutations are mutually exclusive with ATRX mutations. Interestingly, patients with grade II and III *IDHwt* tumors but without a *TERTp* mutations appear to have a better prognosis compared to patients with *TERTp* mutations. Typically, these studies have been lacking the assessment of chromosome 7 and 10q, which most likely would have identified a glioblastoma like chromosomal loss pattern in many of the IDHwt/*TERTp* mutated tumors. More clinical outcome data on these tumors are urgently needed. Assessment of *TERTp* mutational status can be useful for *IDHwt* diffuse glioma, they are however not specific for glioma and for example occur also in medulloblastoma.

The gain of 7 and loss of 10q genotype

The combination of tri/polysomy of chromosome 7 and LOH of 10q is a characteristic combination found in many glioblastoma and probably represents an early event in these tumors.[19, 44] Usually *TERTp* mutations are present, and in 40-50% of cases *EGFR* amplification usually with *EGFR* mutations, including *EGFRvIII* mutations in 20%. Many *IDHwt* astrocytoma and anaplastic astrocytoma (especially in patients over 45 years of age) show this 7+/10q- pattern, and typically have a clinically aggressive course (figure 1b).[6, 45]Testing for this combination in patients over 45-50 years of age with grade II of III *IDHwt* tumors may give positive indications for a poor prognosis. The WHO classification strongly suggests the diagnosis glioblastoma should be considered in 7+/10q- anaplastic oligodendroglioma and anaplastic oligoastrocytoma, but these abnormalities do not qualify for the diagnosis glioblastoma ion the current classification. The available clinical data support that despite these being histologically grade II or III tumors, that these tumors should be treated as glioblastoma and many clinicians with routine access to diagnostics of 7 and 10q do so.

Epidermal Growth Factor Receptor (EGFR) amplification and mutations

EGFR amplification occurs in 40-50% of all glioblastoma, and is usually associated with EGFR mutations and trisomy/polysomy of chromosome 7.[46] Most *EGFR* amplified tumors also show *EGFR* mutations

affecting the extracellular domain of the receptor, the most frequent being the *EGFRvIII* mutation. There is currently no drug that specifically or effectively exploits any of these mutations, although several trails on novel agents are ongoing. As a consequence, from both a therapeutic and a diagnostic aspect the routine assessing of *EGFR* amplification or *EGFR* mutations is currently not useful. The presence of EGFR amplification is indeed highly specific: if found it is diagnostic at the molecular level of a glioblastoma, but it lacks sensitivity: assays for EGFR amplification will be negative in 50% of the glioblastoma cases. Outcome of *EGFR* amplified or *EGFRvIII* mutated tumors is not different from other glioblastoma.[47] Currently, for glioblastoma diagnostics assessing both chromosome 7 and 10q or *TERTp* mutations is more informative than assessing EGFR amplification status.

Phosphatase and Tensin Homolog (PTEN)

PTEN mutations occur in 20-30% of glioblastoma, and are as a rule accompanied by LOH10q. When both are present this results in bi-allelic PTEN inactivation. They may also occur at low frequency in other gliomas with unclear clinical significance, and in other tumors (medulloblastoma). Thus, it has low diagnostic value and no therapeutic consequences. Routine assessment of PTEN mutations is clinically not indicated.

BRAF-KIAA fusion genes and BRAF mutations in glial tumors

Abnormalities in the BRAF gene are characteristic of several subgroup of gliomas. Pilocytic astrocytoma (PA) in the fossa posterior typically have a tandem duplication at 7q34 resulting in a transforming fusion gene between *KIAA1549* and *BRAF* (*BRAF* duplication or *BRAF-KIAA1549* fusion gene), but not the *BRAFv600* mutation. *BRAF-KIAA* fusion genes are also frequent in non-NF1 optic nerve glioma (73%).[48] *BRAF-KIAA*1549 fusion are age specific, they are rare in PA patients over 40 years of age (7%). BRAFv600 mutations are mutually exclusive with the BRAF-KIAA549 fusion gene, these are observed in , 33% of the non-posterior fossa PA. They are also relatively common in pleomorphic xantroastrocytoma (PXA; 43-

66%), anaplastic PXA (65%) and ganglioglioma (18-43%) especially if located in the brain stem[49-52]; they are rare in adult glioma (glioblastoma: 2%, adult low grade glioma: 0-3%).[49] They are also frequent in the proposed novel (but rare) WHO entity of epitheloid glioblastoma, although their distinction from anaplastic PXA is unclear.[53] A study on pediatric diencephalic low grade glioma reported frequent *BRAFv600* mutated non-PA in this region, with imaging characteristics of vivid enhancement and multiloculated or multinodular appearance and/or infiltrative growth on T2 weighted images (figure 2).[54] A Canadian series observed *BRAF* fusion positivity in unilateral thalamic low grade tumors.[55] Since *BRAF* mutated tumors may be treated with targeted agents aiming at the *BRAFv600* mutations, either alone or in combination with a MERK pathway inhibitor, the finding of this abnormality may have therapeutic implications. Responses to these agents have been described, and this appears a very promising avenue of research.[56] *BRAF* mutations and the *BRAF-KIAA* fusion have not been incorporated into the current diagnostic classification; the diagnosis of pilocytic astrocytoma remains a morphological definition. Routine testing must be considered in relevant cases. Future research should focus on establishing to what extent these tumors share the same background, and to what extent other abnormalities in the *RAS/RAF* pathway may have a similar phenotypic effect. More rare genetic lesions in pilocytic astrocytoma include *NF1*, *KRAS* and *RAS* mutations and *FGFR1* and other *BRAF* fusions.[57]

Histone H3F3A and HIST1H3B mutations

The WHO 2016 has accepted the 'diffuse midline glioma, H3 K27M mutant' as diagnostic entity, occurring predominantly in childhood and adolescent brain tumor patients. The mutation is part of a larger family of histone mutations with similar clinical presentation. Pediatric and young adult glioma frequently show mutations in genes encoding H3 variants, which through histone modification alter gene expression.[58] Driver mutations occur in the *H3F3A gene* (positions K27 and G34) encoding histone H3.3 genes, and in *HIST1H3B* histone H3.1 gene (K27 position). K27 mutated tumors typically arise in the

brainstem and midline structures such as thalamus and cerebellum, mostly in children and young adults. Thus, diffuse intrinsic pontine glioma (DIPG) frequently harbor K27M mutations in histone H3.3 genes as well as in H3.1 genes.[59] Childhood and young adult supratentorial glioma may show mutations in histone H3.3, with K27M mutations occurring in midline tumors. In contrast, pG34R/V histone H3.3 mutations are restricted to pediatric and young adult HGGs of the cerebral cortex, and are almost invariably associated with *ATRX* and *TP53* mutations.[59, 60] K27 mutations are associated with a poor outcome; G34 mutations appear to have better survival. Intrinsic pontine glioma harboring a K27M mutation in H3.3 are less responsive to radiotherapy, with earlier relapses and more metastatic recurrences than those in H3.1.[60] Although the K27M mutation was frequently observed in adult brainstem and thalamic gliomas, this mutation tended to be associated with a poorer prognosis in brainstem gliomas but not in thalamic gliomas.[61] The presence of the H3F3A K27M mutation is associated with mutations in *TP53*.[59, 62] An antibody against the K27M allele may prove useful to facilitates detection of this mutation.[63] The role of other mutations (e.g., ACVR1) in DIPG remain to be elucidated. Testing for *H3F3A* mutations is insightful in pediatric and young adult cases with midline tumors.

Challenges: platforms and tests to be used

The revised WHO 2016 criteria do not make recommendations how to assess molecular alterations, which is wise in view of the rapidly changing landscape of molecular diagnostics and testing platforms. With next generation sequencing techniques (NGS) becoming rapidly more affordable, NGS panels tailored for glioma diagnostics are increasingly being used for routine diagnostics including assessment of copy number alterations.[64] Although there is clearly an advantage of the assessment of more than only *IDH* mutation and 1p/19q status, the routine use of screening for the 50 most frequent cancer genes or whole exome in glioma is without clinically proven benefit. Outside the identification of

molecular glioblastoma with WHO grade II or III histology, no proven therapeutic decisions can be taken based on these profiles, with *BRA*F mutant tumors as the most promising exception as they allow patients to be selected for clinical trials.[56] Previous studies have shown the clinical usefulness of gene expression analysis and genome wide methylation analysis. In particular, the latter approach has been shown to be very informative, allowing the classification of tumors without knowledge of specific mutations. This classification system is based on the assumption that the methylation pattern of a tumor is the consequence of both the lineage of the cell the tumor arises from, and tumor specific DNA characteristics. For brain tumors, a relevant aspect here is that the analysis of methylation status simultaneously allows the assessment of *MGMT* status, which may well be the single most powerful determinant of benefit from alkylating agent chemotherapy.[42, 45]

Assessment of molecular characteristics in every day practice and pitfalls

Testing for *IDH* is part of routine diagnostics, but it seems reasonable to limit routine testing to an age range of 15 to 55 - 60 years, for example, and test beyond that only on clinical indications (e.g. in all adult grade II and III glioma, in the presence of oligodendroglial features, and in case of a hemispheric astrocytoma in a 13 year old). For the *R132H* mutation a very reliable immunohistochemistry (IHC) assay is available, but this represents only 90% of all *IDH* mutations. As a consequence, IHC has at best a 90% sensitivity, implying that in case of IHC negativity this must be followed by sequencing for both *IDH1* and *IDH2* mutations (figure 4). IHC can be used as a first screen, but not as a tool to rule out *IDH* mutations. Testing for 1p/19q status and *IDH* mutations should be performed in all patients presenting with possible oligodendroglial tumors. Testing for 1p/9q status should use an assay that allows assessment of loss of the entire 1p and 19q arm. Fluorescence in situ hybridization (FISH) for 1p using a probe for 1p36.6 region is less specific as it may suggest loss in tumors with partial 1p deletion only, limited to the tip of chromosome 1p.[65, 66] This part can be lost without loss of the rest of chromosome 1p, which in

combination with 19q loss has been observed in glioblastoma. If copy number alterations (CNA) are considered to be relevant this should be assessed with other techniques. Both 1p/19q co-deletions and IDH mutations are early events in gliomagenesis, and their presence or absence are unlikely to change over time.[67, 68] Therefore, retesting of 1p/19q and *IDH* status at the time of a re-resection in tumors with already known status is of limited use, unless a significant clinical change occurred indicating a second tumor. Incorporation of assessment of *TERTp* mutations into routine diagnostics of gliomas has been suggested.[20] In the absence of 1p/19q loss, diffuse gliomas with *TERTp* mutations tend to have a poor outcome reminiscent of glioblastoma.[7, 43] Although some studies on targeted mutation assessment have shown that in some tumors only *TERTp* mutations were observed, this deserves further clinical study since in most of these series tumors were not tested for CNA of 7 and 10q.

Reporting

Centers must develop automated workflows that incorporate molecular testing in their routine procedures, including the incorporation of the molecular diagnostics in the final pathology result. It is important for the reporting of the diagnosis to be standardized and made available for capture in the national cancer registry databases, so the incidence of the specific entities based on molecular features can be reported. Since the turnaround time for histopathology is shorter than for molecular diagnostics, ensuring accurate and timely feedback on molecular findings in patients in whom a histopathological diagnosis has already been established is important. It is recommended to routinely include (MR) imaging characteristics in the final diagnostic considerations: MRI images should be consistent with the pathological diagnosis and if not this should give rise to additional scrutiny. The interpretation of molecular findings depends on the context: if the tumor is unlikely to be a diffuse glioma the molecular findings may not contribute except when an diagnostic mutation is found with supportive MR and clinical findings.

Clinical studies

With the new classification, the clinical data from past trials without molecular analysis have become outdated. Since the first results of the trials on anaplastic oligodendroglioma, follow-up trials in the newly diagnosed setting (CODEL, CATNON) but also in recurrent disease (TAVAREC) enrolled patients based on their 1p/19q status because of the difference in prognosis, and many prospective trials reported retrospectively on the molecular status (table 3). New studies should now distinguish between IDH mutant tumors, IDH mutant and 1p/19q co-deleted, and IDH wild type diffuse gliomas. This complicates matters. As an example, the presence of IDH mutations also identifies a more favorable subgroup of glioblastoma which may also hold true at the time of recurrence.[69] This questions whether these tumors should be enrolled in trials on recurrent glioblastomas and whether it should be routinely tested for. On the other hand, the still modest survival of IDH mutated glioblastoma also argues against enrolling these tumors in trials aiming at IDH mutated diffuse grade II and III glioma although the limited difference in outcome between grade II and III IDH mutated tumors provides a rational for combining these grades.[15] Today's changes emphasize that all trials should collect tissue samples as part of the study design. Analysis of existing datasets may help to improve our understanding of the outcome of these subsets of patients.

Quality control

The interobserver variation in the histopathological classification of glioma is well known, but early experiences with inter-laboratory tests on diagnostic molecular assays on the same set of tumors revealed that differences between laboratories may exist as well.[70, 71] Proper quality control is critical now that diagnostics and clinical decisions are based on molecular testing. Laboratories need to certify and validate their testing procedures with appropriate controls. This is not exciting work and requires significant efforts, but is absolutely essential for reliable diagnostics.

Conclusion.

The new WHO 2016 classification for brain tumors brings molecular diagnostics to the center of glioma classification. This revised classification will improve treatment selection of brain tumor patients and clinical trial design. This will not be the last revision of this classification as new molecular insights into brain tumors will further refine the classification of brain tumors. Further refinements already seem indicated, e.g., in the IDH wild type categories of grade II and III glioma as these represent in many cases –especially in patients over 50 years of age- glioblastoma -like lesions with 7+/10q LOH. Further analysis of *TERTp* mutational status in non-glioblastoma 1p/19q intact tumors will be needed to better understand the prognostic role of that mutation in diffuse glioma. More responsiveness to the rapidly changing and multidisciplinary field of neuro-oncology will be crucial to maintain a well-accepted WHO classification of brain tumors. For this, a more transparent and multidisciplinary process of change of these pivotal criteria will be needed.

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Figures

Figure 1 Glioblastoma diagnostics anno 2016. 1a. T1 weighted MR images of a 40 year old male with a short history headache and difficulty walking . At histopathology a glioblastoma was diagnosed, targeted sequencing showed an *IDH2* mutation, combined 1p/19q loss and deletion of chromosome 9 consistent with the diagnosis of an anaplastic oligodendroglioma; 1b. T1 weighted contrast enhanced MR image of a 50 year old female who developed over months progressive memory and behavioral complaints. No contrast enhancement was present, at biopsy histopathology showed a grade II astrocytoma, Next Generation Sequencing failed to show an *IDH* mutation but instead documented gain of chromosome 7, loss of 10q, and mutations in the EGFR and PTEN gene consistent with a glioblastoma.

Figure 2. T2 weighted images of a 25 year old male who underwent two biopsies for a mesencephalic lesion, on both occasions histopathological examination failed to show clear evidence of tumor. On mutational analysis, a *BRAF* mutation was found (c.1795_1797dupACA;p.T599dup)

Figure 3. T1 contrast enhanced MR images of a ring right frontal enhancing lesion tumor histopathologically classified as glioblastoma, at molecular examination 1p/19q co-deletion was found but with neither an *IDH* nor a *TERTp* mutation,

Figure 4. T2 weighted MR images of a right frontal low grade astrocytoma in a 20 year old, diagnosed as *IDH wt* because of negative immunohistochemistry. After referral to a tertiary care center sequencing demonstrated an *IDH* c.394C>T; p.R132C mutation in combination with a *TP53* and an *ATRX* mutation.

Table 1. The WHO 2016 classification for astrocytoma, oligodendroglioma and ependymoma and ICD10 code.

WHO 2016 classification of astrocytoma, oligodendroglioma and ependymoma	
Diffuse astrocytoma and oligodendroglial tumors	ICD code
Diffuse astrocytoma, IDH mutant	9400/1
Gemistocytic astrocytoma, IDH mutant	9411/3
Diffuse astrocytoma, IDH wildtype	9400/3
Diffuse astrocytoma, NOS	9400/3
Anaplastic astrocytoma, IDH mutant	9401/3
Anaplastic astrocytoma, IDH wild type	9401/3
Anaplastic astrocytoma. NOS	9401/3
Glioblastoma, IDH wild type	9440/3
Giant cell glioblastoma	9441/3
Gliosarcoma	9442/3
Epitheloid glioblastoma	9440/3
Glioblastoma, IDH mutant	9445/3
Glioblastoma, NOS	9440/3
Diffuse midline glioma, H3 K27M-mutant	9385/3
Oligodendroglioma, IDH mutant and 1p/19q co-deleted	9450/3
Oligodendroglioma, NOS	9450/3
Anaplastic oligodendroglioma, IDH mutant and 1p/19q co-deleted	9451/3
Anaplastic oligodendroglioma, NOS	9451/3
Oligoastrocytoma NOS	9382/3
Anaplastic oligoastrocytoma	9382/3
Other astrocytic tumors	
Pilocytic astroctyoma	9421/1
Pilomyxoid astrocytoma	9425/3
Subependymal giant cell astrocytoma	9424/3
Anaplastic pleomorphic xanthoastrocytoma	9424/3
Ependymal tumors	
Subependymoma	9383/1
Myxopapillary ependymoma	9394/1
Ependymoma	9391/3
Papillary ependymoma	9393/3
Clear cell ependymoma	9391/3
Tancytic ependymoma	9391/3
Ependymoma, RELA fusion positive	9396/3
Anaplastic ependymoma	9392/3

Table 2

Table 2. Survival in various molecular subtypes as reported in randomized controlled clinical studies with molecular analysis and mature overall survival results.

study	histology	Molecular subtype	treatment	n	Median OS	Median PFS
RTOG 9802[72]	Low grade glioma	*IDH* mutated (all) *IDHwt*	RT/PCV or RT RT/PCV or RT	71 42	13.1 yrs 5.1 years	
EORTC 26951[6, 73]	Anaplastic oligodendroglioma	1p/19q codeleted I*DHmt* 1p/19q intact 7+/10q-/*TERTpmt*	RT/PCV RT/PCV RT or RT/PCV	43 23 55	NR (>14 yrs) 8.3 yrs 1.13 yrs	147 4.2 yrs NS
RTOG 9402[74]	Anaplastic oligodendroglioma	1p/19q I*DHmt* (all)	RT/PCV	59	14.7 yrs	8.4 yrs
RTOG 9804[75]	Anaplastic astrocytoma	*IDH mt* (IHC) I*DHwt*	RT/chemo	49 54	7.9 yrs 2.8 yrs	
NOA4[76]	Grade III	1p/19q codeleted *IDHmt* 1p/19q intact I*DHwt*	RT or chemo	66 83 58	NR 7.0-7.3 yrs 3.1 – 4.7 yrs	

Figure 1a, b

Click here to download Figure figure 1a_b_glioblastoma_1p19q idh.tif

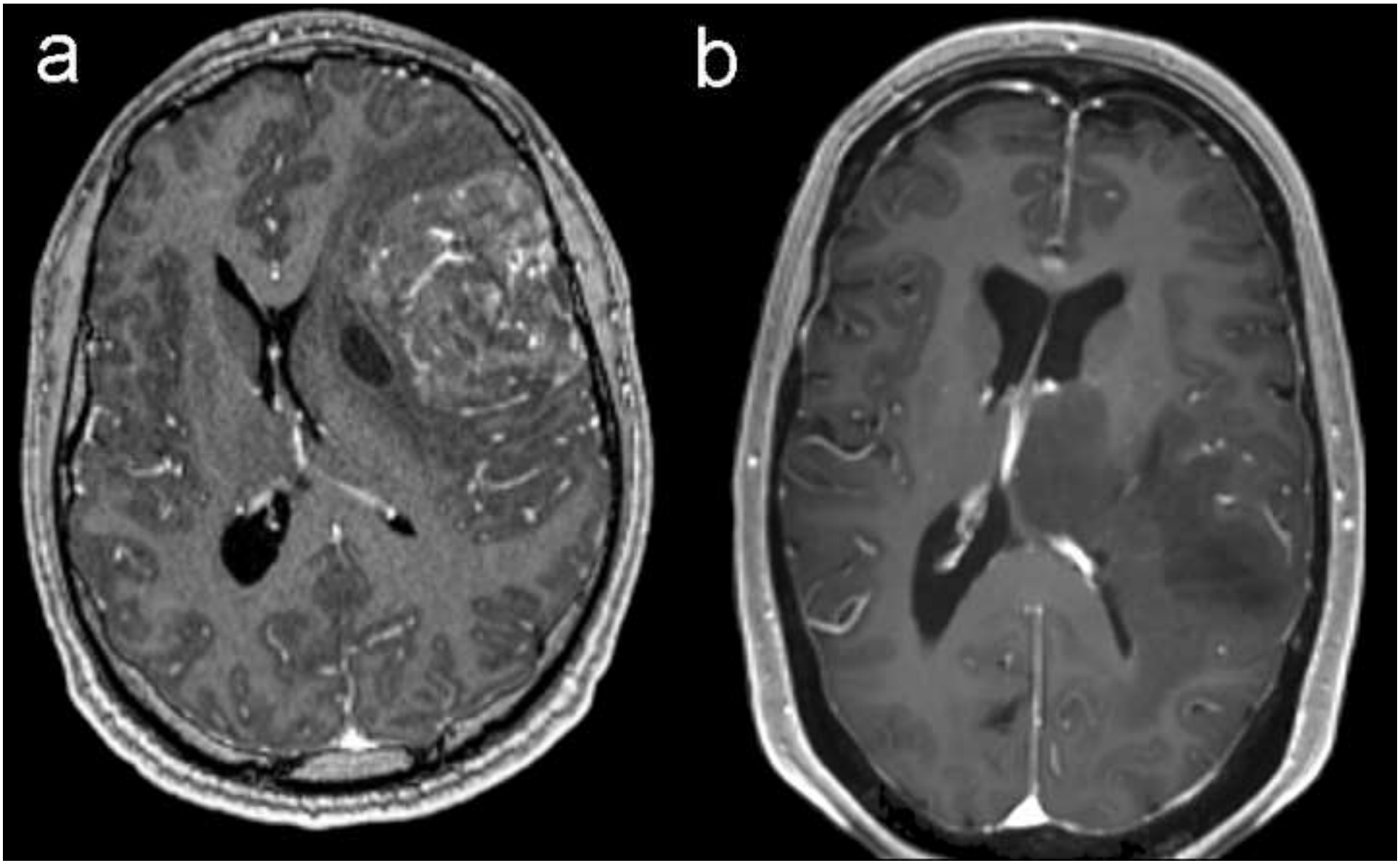

Figure 2

Click here to download Figure fig 2_T2_BRAFmt.tif

Figure 3

Click here to download Figure fig 3_1p19q deleted no IDHmt.tif

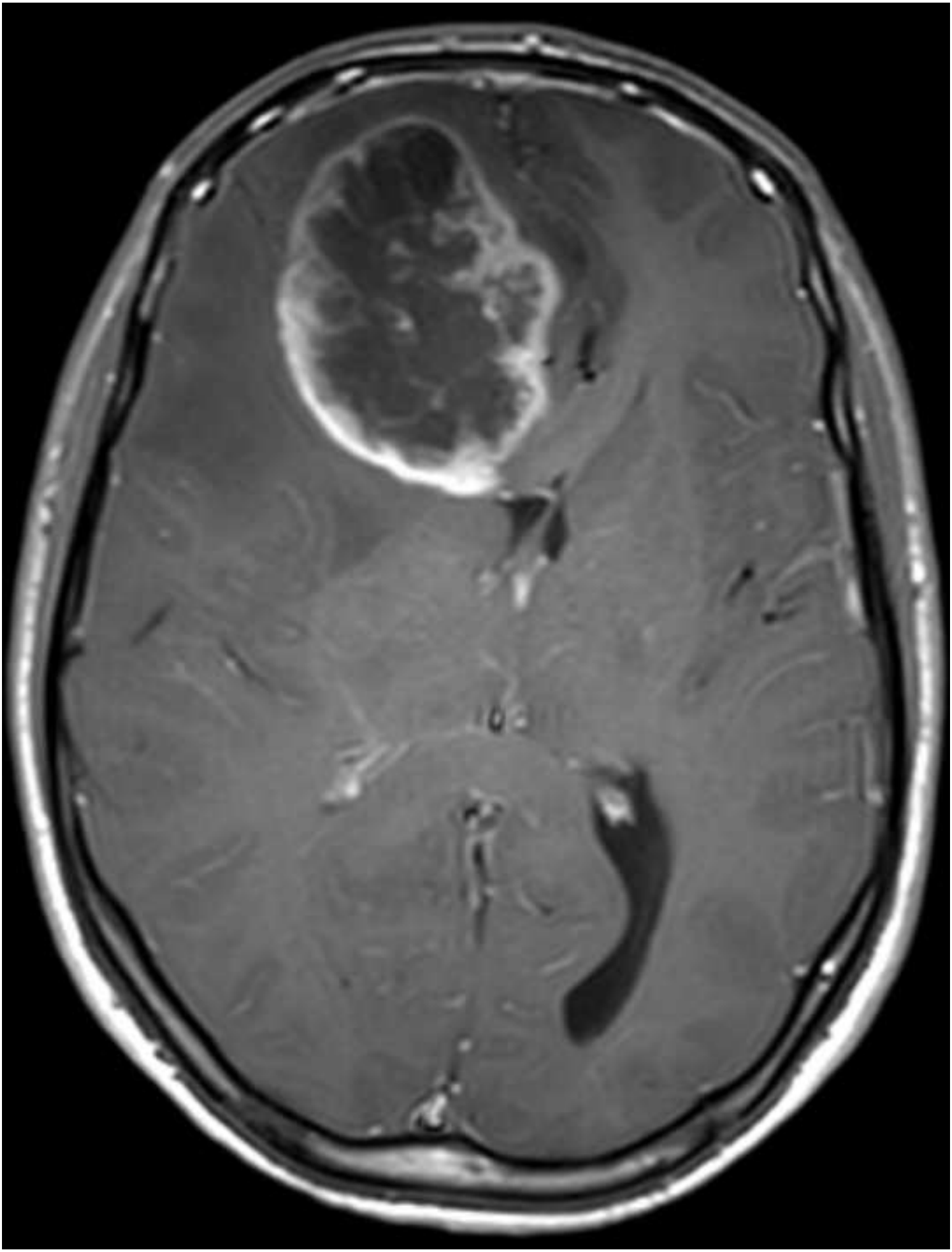

Figure 4

Click here to download Figure fig 4_T2 IDH IHC neg seq IDH mut pos.tif